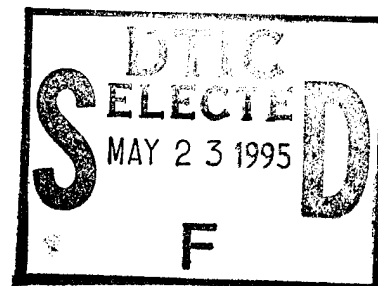


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Amphetamine Redistributes Dopamine from Synaptic Vesicles
to the Cytosol and Promotes Reverse Transport

by

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Amphetamine Redistributes Dopamine from Synaptic Vesicles to the Cytosol and Promotes Reverse Transport

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Abstract

Whether amphetamine acts principally at the plasma membrane or at synaptic vesicles is controversial. We find that *d*-amphetamine injection into the *Planorbis* giant dopamine neuron causes robust dopamine release, demonstrating that specific amphetamine uptake is not required. Arguing for action at vesicles, whole cell capillary electrophoresis of single *Planorbis* dopamine neurons shows that amphetamine reduces vesicular dopamine, while amphetamine reduces quantal dopamine release from PC12 cells by >50% per vesicle. Intracellular injection of dopamine into the *Planorbis* dopamine neuron produces rapid nomifensine-sensitive release, showing that an increased substrate concentration gradient is sufficient to induce release. These experiments indicate that amphetamine acts at the vesicular level where it redistributes dopamine to the cytosol, promoting reverse transport and dopamine release.

Key words:

amphetamine, reverse transport, synaptic vesicles, PC12 cells, *Planorbis* corneus, amperometry, electrochemistry

Amphetamines are dopamine (DA) transporter substrates that induce calcium-independent monoamine release. Exactly how this occurs has been controversial (Seiden et al., 1993). In the exchange diffusion model, the DA transporter is postulated to have a mobile site that mediates the translocation of DA across the membrane. *d*-Amphetamine (AMPH) is thought to act by substituting for DA, driving the carrier to the internal face of the plasma membrane where the site binds cytoplasmic DA and then releases it on returning to the external face (Fischer and Cho, 1979). Although monoamine uptake transporters have been sequenced (Amara and Kuhar, 1993; Giros and Caron, 1993) it is not clear whether their putative structures define a channel or a carrier (Levi and Raiteri, 1993).

In the weak base model (Sulzer and Rayport, 1990; Sulzer et al., 1993), AMPH-like psychostimulants are thought to redistribute catecholamines from synaptic vesicles to the cytosol by collapsing the vesicular proton gradient that provides the free energy for neurotransmitter accumulation (Ereckinska et al., 1987; Sulzer and Rayport, 1990; Rudnick and Wall, 1992). This would increase the availability of transmitter for reverse transport without requiring a mobile site. In support of this model, weak bases and protonophores, which cause an AMPH-like release of DA from isolated vesicles (Sulzer and Rayport, 1990) but are not substrates for the uptake transporter, induce reverse transport in cultured DA neurons (Sulzer et al., 1993). In addition, low affinity binding of AMPH to the vesicular transporter (Gonzalez et al., 1993; Schuldiner et al., 1993) may block vesicular uptake and cause a redistribution of DA similar to that mediated by collapse of synaptic vesicle proton gradients.

The inhibition of AMPH action by uptake blockers including cocaine and nomifensine (Fischer and Cho, 1979; Liang and Rutledge, 1982; Hurd and Ungerstedt, 1989) suggests that uptake of AMPH as a substrate is required. However, it is not clear whether AMPH uptake alone is sufficient or whether AMPH acts intracellularly to promote DA release. To differentiate between the plasma membrane and synaptic vesicles as the principal site of AMPH action, we have used identified DA neurons to ask whether injections of AMPH, which bypass the uptake transporter, cause DA release. Then to clarify the mode of action, we have used recently developed microelectrochemical techniques to measure AMPH-induced redistribution of DA across the vesicular and plasma membranes. Finally, we have addressed the role of the plasma membrane transporter by monitoring DA release due to intracellular DA injection in real time.

Materials and Methods

GDC electrochemistry

The use of the *Planorbis corneus* giant dopamine cell (GDC) for electrochemical experiments has been described previously (Chien et al., 1990; Ewing et al., 1992). Briefly, adult *Planorbis corneus* were obtained from NASCO (Fort Addison, WI) and dissected to reveal the left pedal ganglion, which was immersed in 10 mL of snail saline and desheathed to expose the GDC. Snail saline contained 39 mM NaCl, 1.3 mM KCl, 4.5 mM CaCl₂, 1.5 mM MgCl₂, 6.9 mM NaHCO₃, pH 7.4. Intracellular injections of AMPH or DA were carried out with pulled glass capillaries (0.5 μ m tips) and an Eppendorf Model 5242 pressure-based microinjector. Injection volumes were calibrated by injection of aqueous solution into mineral oil under microscopic observation and calculation of the volume of

the spherical droplet. For AMPH injections, a 2 μm carbon ring ultramicroelectrode touching the GDC membrane was held at a steady potential of 0.8 V and the oxidation current measured over time. For DA injection experiments, the carbon ring electrode was again placed immediately against the GDC. Staircase voltammetry (-0.2 to 0.8 V, 50 steps, 20 mV/step, 50 ms/step) was used throughout to monitor DA release; current was sampled from each plotted voltammogram at 0.8 V vs. a sodium-saturated calomel reference electrode and plotted with respect to time. Nomifensine was applied by bath perfusion. All experiments were conducted at room temperature.

Single cell capillary electrophoresis

To measure the vesicular DA pool, single GDCs from juvenile snails were drawn into 25 μm i.d. x 75 cm long capillaries (Olefirowicz and Ewing, 1990; Kristensen et al., 1994). A 1 min injection of morpholinoethanesulfonic acid buffer (pH 5.65) was timed to produce an optimal differential lysis of the plasma but not vesicular membranes. The resulting cellular components were then separated by electrophoretic mobility in a 25 kV field applied to the capillary and detected using a carbon fiber electrode held at 0.8 V.

PC12 cell quantal analysis

PC12 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained using established methods (Greene and Tischler, 1976). The catecholamine present in these cultures consists mostly of dopamine (about 90%); the remainder is mostly norepinephrine (E. Pothos and D. Sulzer, unpublished observations). Recording media contained 150 mM NaCl, 4.2 mM KCl, 1.0 mM

NaH₂PO₄, 11.2 mM glucose, 0.7 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, (pH 7.4). Amperometric detection of individual exocytic events were monitored with a 5 μ m diameter carbon fiber electrode (Kelly and Wightman, 1986) using an EI-400 potentiostat (Ensmann Instrumentation, Bloomington, IN) and recorded to disk (Gateway 2000 PC) with a LabMaster Interface (Scientific Solutions, Solon, OH). The working electrode was placed on a target cell and gently pushed down against the cell body (about 1 μ m) with a piezo-micropositioner (PCS-750/1000, Burleigh Instruments, Fishers, NY). Micropipettes containing stimulants were placed 10 μ m above the substrate and 150 μ m away from the target cell by micropositioners (Mertzhauser, Germany). Chemical stimulants were delivered by 6 s pressure pulses from a microinjector (Picospritzer II, General Valve, Fairfield, NJ). The estimated total injection volume was 30 nL per injection. Stimulation was carried out with a mixture of 105 mM KCl and 1 mM nicotine as this was found to produce more exocytic events than elevated potassium or 1 mM nicotine applied separately.

The output from the potentiostat was digitized at 820 Hz and analyzed using a locally written program (Chen et al., 1994). In short, a noise threshold was set as a slope of 1.4 x the average slope of the 60 Hz noise (18 consecutive 60 Hz peaks). Peaks were defined as beginning at the starting point of any four consecutive data points with positive slopes greater than the noise threshold. The ends of the peaks were defined as either the first point of four consecutive data points with a positive slope immediately after a negative slope or the value of the starting point of the peak, whichever was higher. Only peaks with baseline width between 9 to 29 ms (about 99% of the peaks identified) were considered in order to eliminate very rapid current transients or rare wide peaks that could represent overlapping multiple events. The number of molecules oxidized at the electrode

face was determined by the relation $N = Q/nF$, where Q is the charge of the peak, n was assumed to be 2 (for catecholamines), N is the number of moles and F is Faraday's constant (96,485 coulombs per equivalent).

Results

Intracellular AMPH injection and DA release

The left pedal ganglion of the pond snail *Planorbis corneus* contains a single giant DA neuron (GDC) that closely resembles mammalian DA neurons in several ways (Osborne et al., 1975). The GDC displays nomifensine-sensitive DA uptake, reserpine-sensitive vesicular DA uptake, and metabolizes DA to dihydroxyphenylacetic acid (DOPAC). However, unlike mammalian DA neurons, the adult GDC is approximately 200 μm in diameter, facilitating single cell analysis; and, it lacks ascorbic acid (Barber and Kempter, 1986), facilitating the unambiguous electrochemical detection of DA.

We measured DA release in real time with carbon fiber microelectrodes (Wightman et al., 1991) touching the extracellular face of the GDC membrane (Fig 1A). Injection of 8 pL of 100 mM d-AMPH into the GDC rapidly released DA (Fig 1B). Based on an estimated cytosolic volume of 8 nL, the injection resulted in an intracellular concentration of about 100 μM AMPH. Although AMPH is amphiphilic and may diffuse from the cell, it also diffuses rapidly in aqueous medium and would reach nanomolar levels within milliseconds. Extracellular DA increased following the injection, reaching a maximum of $1.43 \pm 0.58 \mu\text{M}$ (mean \pm s.e.m., $n = 3$). The shape of a voltammogram taken at the peak current value closely matched the voltammogram of a DA standard (Fig 1C).

Measurement of the vesicular DA pool

To examine the effects of AMPH on the vesicular pool directly, we measured vesicular DA by capillary electrophoresis of juvenile GDCs (75 μm in diameter) (Kristensen et al., 1994). Time-limited membrane lysis of single GDCs produces two distinct DA peaks (Fig 2 lower trace; cf. Kristensen et al., 1994). Peak 1 is narrow and appears at the same time as a DA standard, indicating that it reflects free DA and DA from a readily releasable vesicular pool. Peak 2 appears later and is broader; furthermore, it is abolished by prior reserpine treatment or by more extensive lysis, indicating that it reflects a slower release of DA during electrophoresis from vesicles that have not undergone exocytosis. Thus Peak 2 provides a measure of vesicular DA principally in storage vesicles.

We found that prior treatment with AMPH (10 μM for 15 min) reduced Peak 2 to below detectable limits and correspondingly increased Peak 1 (Fig 2 upper trace; Table).

AMPH modulation of quantal size

To measure changes in the DA content of individual secretory vesicles, we monitored individual catecholamine release events (Wightman et al., 1991) in undifferentiated PC12 cells (Chen et al., 1994), where release occurs from the cell body. Quantal packets were measured with a detection limit of 19,000 molecules. Since spontaneous release events in PC12 cells were rarely observed and undifferentiated PC12 cells do not produce action potentials, the cells were stimulated by local perfusion with nicotine and KCl (Chen et al., 1994). In each case, release events were measured for approximately two minutes following

stimulation. In controls, individual exocytic events corresponded to the release of $111,000 \pm 32,000$ catecholamine molecules ($n = 9$ cells, 92 ± 37 events per cell, mean \pm s.e.m.). AMPH ($10 \mu\text{M}$ for 10 min) reduced stimulated catecholamine released per exocytic event. Randomly chosen amperograms for exocytosis from control cells and those treated with AMPH are shown in Fig 3A and 3B. When paired with age matched controls from sister cultures ($n = 3$, 139 ± 105 events per cell), the average exocytic event following incubation with AMPH ($n = 7$ cells, 34 ± 9 events per cell) decreased to $48 \pm 5.4\%$ of control (Fig 3C); very few vesicles releasing $> 200,000$ molecules were present following AMPH.

DA release due to intracellular DA injection

To determine whether increased cytosolic DA is sufficient to induce release, we returned to the GDC. In the resting state, cytosolic DA is about $2.2 \mu\text{M}$ and extracellular DA below the limits of detection (Ewing et al., 1992). Intracellular injection of 4 pL of 0.5 mM DA (Fig 4A) rapidly increased extracellular DA to $22.5 \pm 0.9 \mu\text{M}$, 3.5 s post-injection ($n = 3$ cells); extracellular perfusion with nomifensine (a specific blocker of the DA uptake transporter that, unlike other commonly used uptake blockers, has no detectable effect on intracellular proton gradients; Sulzer et al., 1993) attenuated the increase in extracellular DA following subsequent injections by $87 \pm 4\%$. After removal of nomifensine, the increase in extracellular DA due to intracellular DA injection returned to near control levels ($20.4 \pm 1.5 \mu\text{M}$ peak concentration, $p = 0.265$ by Student's t-test, not significantly different). Intracellular injections of control solution lacking DA induced no DA release (data not shown). Difference voltammograms taken after the first, second, and fourth DA injections (Fig 4B) closely resembled voltammograms of DA obtained by *in vitro* calibration.

DISCUSSION

Whether AMPH acts principally at the plasma membrane uptake transporter or at the vesicular level has been controversial. We used two model systems to address this issue. Arguing for a principal mechanism of action at the vesicular level, intracellular injection of AMPH into the *Planorbis* GDC, which bypasses the plasma membrane uptake transporter, causes robust DA release. Whole cell capillary electrophoresis of GDCs shows that AMPH profoundly reduces vesicular DA; and amperometric detection of DA release in PC12 cells shows that AMPH significantly reduces DA release from individual release events. Taken together, these results indicate that amphetamine redistributes DA from synaptic vesicles to the cytosol. Monitoring DA at the extracellular face of the GDC plasma membrane shows that intracellular injection of DA produces rapid nomifensine-sensitive release, indicating that increased cytosolic DA is sufficient to trigger reverse transport.

AMPH acts intracellularly

Injection of AMPH directly into the cytosol of the GDC causes DA release even though the uptake transporter is bypassed and the AMPH concentration gradient is in the opposite direction from that required for net efflux via exchange diffusion. Together with the uptake of AMPH by catecholamine transporters (Zaczek et al., 1991) which would play a role in the specificity of transmitter release, and the ability of AMPH to release monoamines from isolated vesicles (Slotkin and Kirshner, 1971; Knepper et al., 1988; Sulzer and Rayport, 1990), these observations argue for an intracellular site of AMPH action.

Consistent with redistribution of DA from vesicles to cytosol, capillary electrophoresis of single GDCs shows that AMPH redistributes DA from the putative vesicular DA pool. The redistribution of DA is greater than that from isolated vesicles (Sulzer and Rayport, 1990), perhaps because intracellular AMPH concentrations are increased due to specific uptake (Zaczek et al., 1991).

Moreover, the magnitude of the increase in free DA apparently exceeds the reduction in the vesicular peak. This could result either from blockade of DA metabolism due to AMPH-mediated monoamine oxidase inhibition or from elevated DA synthesis. Alternatively, the disproportionate increase in free DA could be due to lysis of vesicles, leading to an underestimate of the vesicular pool and an overestimate of free DA. Nonetheless, the intact vesicular peak provides a measure of the major fraction of vesicular DA, and its profound reduction by AMPH favors an action of AMPH at the vesicular level. A number of reports on the effects of reserpine on AMPH-induced release (Parker and Cubeddu, 1986; Seiden et al., 1993; St. Remy et al., 1994) have been used to argue for and against models requiring AMPH action at the vesicular level. The present capillary electrophoresis results support a role for a vesicular pool in AMPH-induced DA release.

Furthermore, monitoring individual exocytic release events in undifferentiated PC12 cells, where vesicular release from the cell body can be measured, shows that AMPH reduces the transmitter content of individual vesicles. This occurs with 10 μ M AMPH, a level reached in the brain with a standard dose (1 mg/kg) (Melega et al., 1992), and shows for the first time a reduction in quantal size (greater than 50%) due to a self-administered drug. In conjunction with the well established role of AMPH in promoting stimulation-independent catecholamine

release, these findings demonstrate that AMPH can attenuate stimulus-dependent release by reducing quantal size. These experiments do not, however, differentiate between collapse of the proton gradient (Sulzer and Rayport, 1990) and blockade of the vesicular amine transporter (Reith et al., 1993; Schuldiner et al., 1993), which binds AMPH with low affinity (Gonzalez et al., 1994), and it may be that both effects play roles. Taken together, these experiments argue that AMPH principally redistributes DA from synaptic vesicles to the cytosol.

Alterations of the DA packaged in individual vesicles would be expected to profoundly affect synaptic transmission. Quantal analysis studies generally assume that the packaging of neurotransmitter per synaptic vesicle is invariant (Redman, 1990). However, the present evidence, in conjunction with prior electrochemical results showing considerable variability in the size of release events (Wightman et al., 1991; Chow et al., 1992), suggests that alterations of quantal size could play an important role in the modulation of catecholamine release.

Elevated cytosolic DA induces reverse transport

Injection of DA into the cytosol of GDCs leads to a rapid release of DA that is nomifensine-sensitive. Uptake transport blockers like nomifensine are thought to block transport by binding to the extracellular face of the DA transporter; however, unlike AMPH, they are not transported (Anderson et al., 1989; Ritz et al., 1990). We have previously shown in DA neuron cultures that nomifensine blocks DA release induced by AMPH, weak bases and protonophores (Sulzer et al., 1993), arguing that nomifensine blocks DA reverse transport. Since in the

present experiments nomifensine blocks substrate flux across the DA transporter, most DA release following intracellular injection occurs via reverse transport.

While intracellular injections of DA produce maximal extracellular levels within 3.5 s (the earliest data point), AMPH injection induces maximal DA release more slowly (compare Figs 1B and 4A). This slower action of AMPH would be consistent with a rate-limiting step of AMPH-mediated redistribution of DA from synaptic vesicles to the cytosol. Altered substrate plasma membrane concentration gradients due to vesicular AMPH sequestration could further affect DA flux across the transporter. The extent to which AMPH increases cytosolic DA will depend in part on the kinetics of reverse transport; since reverse transport due to DA is more rapid, increased AMPH-induced cytosolic DA levels are likely to be short-lived.

The present experiments show transporter-mediated release from neurons in real time. This release can be induced solely by an increased transmitter concentration gradient without alteration of transmembrane ionic gradients. The release reaches physiologically-significant levels since the extracellular DA measured in these experiments greatly exceeds the binding constants of DA receptors (O'Dowd et al., 1994). Previously, reverse transport from retinal neurons was demonstrated using GABA uptake blocker-dependent currents in postsynaptic neurons (Schwartz, 1987) and in glial cells using voltage clamp techniques modeled to represent uptake-dependent potassium flux (Szatkowski et al., 1990). More generally, reverse transport appears to be an important alternate form of neurotransmitter release (Levi and Raiteri, 1993). Not only do uptake blockers attenuate calcium-independent release of radiolabeled monoamines (Lonart and Zigmond, 1991; McKenna et al., 1991), but they also

attenuate release of GABA (Haycock et al., 1978; Pin and Bockaert, 1989; Bernath and Zigmond, 1990; Dunlop et al., 1991; O'Malley et al., 1992; Hitri et al., 1994), and glutamate or aspartate (Dunlop et al., 1992; Turner et al., 1992). Reverse transport of GABA appears to mediate signaling between retinal horizontal and cone cells (Schwartz, 1987), reverse transport of glycine appears to modulate hippocampal NMDA receptors (Adam-Vizi, 1992; Attwell et al., 1993), and reverse transport of glutamate during ischemia appears to be an important mode of release (Adam-Vizi, 1992; Attwell et al., 1993). Our results now add a role for reverse transport in AMPH-induced DA release.

Conclusions

These findings demonstrate in invertebrate DA neurons and PC12 cells that AMPH decreases vesicular DA content, presumably resulting in DA redistribution to the cytosol. Cytosolic DA is in turn rapidly released by reverse transport. Since AMPH-induced DA release occurs independently of specific AMPH uptake by the plasma membrane transporter and can be induced in isolated monoamine vesicles (Slotkin and Kirshner, 1971; Knepper et al., 1988; Sulzer and Rayport, 1990), the present findings favor the release of DA from the vesicular pool.

This mechanism has important implications for neurotransmission and pathology. It suggests that AMPH affects synaptic transmission both by increasing activity-independent reverse transport and by decreasing the amount of transmitter released during activity-dependent vesicular exocytosis. The result should be a reduction in the signal-to-noise ratio of stimulation-dependent monoamine synaptic transmission. Furthermore, this redistribution of DA from

the relatively reducing environment of the synaptic vesicle to the cytosol appears to cause local increases in intracellular oxidative stress in DA neuron axons (Cubells et al., 1994) that may initiate AMPH neurotoxicity.

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FIGURE LEGENDS

Figure 1. Intracellular injection of AMPH promotes DA release.

A. Single GDCs from adult *Planorbis* corneus were impaled with an AMPH containing pipette. Oxidation current was measured with a 2 μ m carbon ring ultramicroelectrode touching the GDC membrane and held at a steady potential of 0.8 V.

B. AMPH (8 pL of 100 mM) was injected intracellularly (arrow), thereby bypassing the uptake transporter, and the increase in extracellular DA measured. Similar levels of DA release were found in three replications of the experiment using DC amperometry. The small, slow baseline decrease during these experiments results from adsorption of electrode oxidation products to the electrode surface (Lau et al., 1991).

C. The shape of the voltammogram (-0.2 initial potential to 0.8 V final potential) taken at the peak current value 14 s post injection (filled circles) closely matched the voltammogram for a DA standard (solid line). AMPH, which lacks hydroxyl groups, is not oxidized at these potentials. Calibration: $i = 0.5$ for the experiment and 31.5 for the standard.

Figure 2. Electrophoretic separation of readily oxidized components from a single *Planorbis corneus* giant DA neuron.

Single neurons (75 μ m diameter) from juvenile snails were drawn into capillary tubes. Subsequent injection of morpholinoethanesulfonic acid buffer (pH 5.65) for 1 min produced a partial lysis of the cell membranes, presumably mainly the plasma membrane. Based on this differential lysis, Peak 1 reflects free DA and

DA from easily released vesicles and Peak 2 reflects vesicular DA (see text); Peak 3 reflects other oxidizable compounds with a net neutral charge (Kristensen et al., 1994). AMPH treatment increased Peak 1 and reduced Peak 2 to undetectable levels (see Table).

Figure 3. AMPH exposure decreases the quantal amplitude of individual release events from PC12 cells.

Randomly-chosen electrochemical records of quantal release for (A) a control cell and (B) a cell that has been incubated in 10 μ M d-AMPH for 10 min. Cells were stimulated by local perfusion (30 nL of 1 mM nicotine in 105 mM KCl saline) to induce vesicular exocytosis. C. A histogram shows the percentage of peak sizes in paired control and AMPH-treated cells. Peaks are combined in intervals of 30,000 molecules. The lower limit of each bin size is shown on the abscissa.

Figure 4. Intracellular injection of DA induces reverse transport.

A. Intracellular injection of 4 pL of 0.5 mM DA (arrows) reliably increases extracellular DA. During extracellular perfusion with 10 μ M nomifensine (dashed line) the DA release due to the same DA injections was markedly attenuated. Perfusion with nomifensine-containing medium and its replacement by control medium produce current spikes (asterisks). These data have been baseline corrected by subtracting a curve fitted to the background signal.

B. Background subtracted voltammograms (from -0.2 to 0.8 V) for the first (\bullet), second (—) and fourth (—) intracellular DA injections shown in A. The voltammograms monitored at the face of the neuron are similar to DA. Although the shapes of the voltammograms differ slightly at potentials above 0.5 V, all

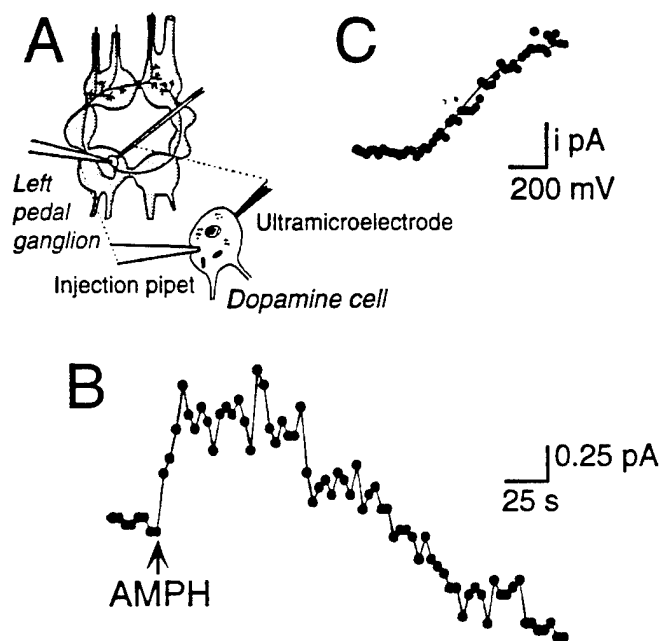
three voltammograms are sigmoidal with a half-wave potential of approximately 0.2 V.

TABLE

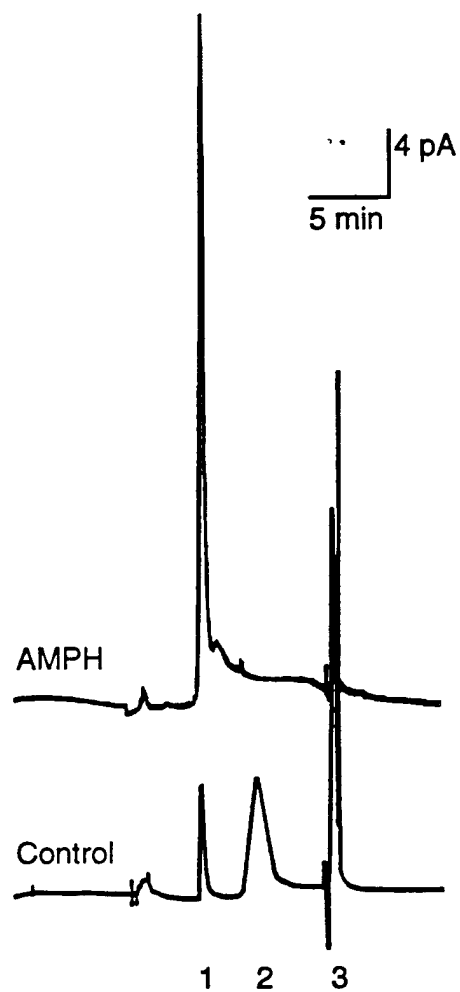
Reduction of Vesicular DA by Amphetamine in an Identified Neuron

	<u>Control</u>	<u>AMPH</u>
Peak 1	6.1 ± 3.0	107 ± 23
Peak 2	19 ± 9.1	< 0.01

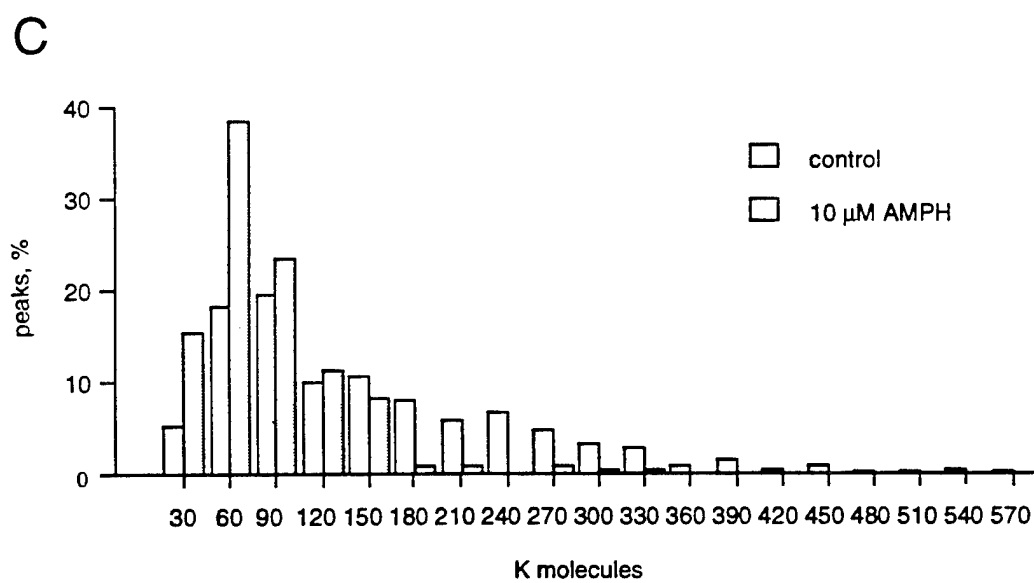
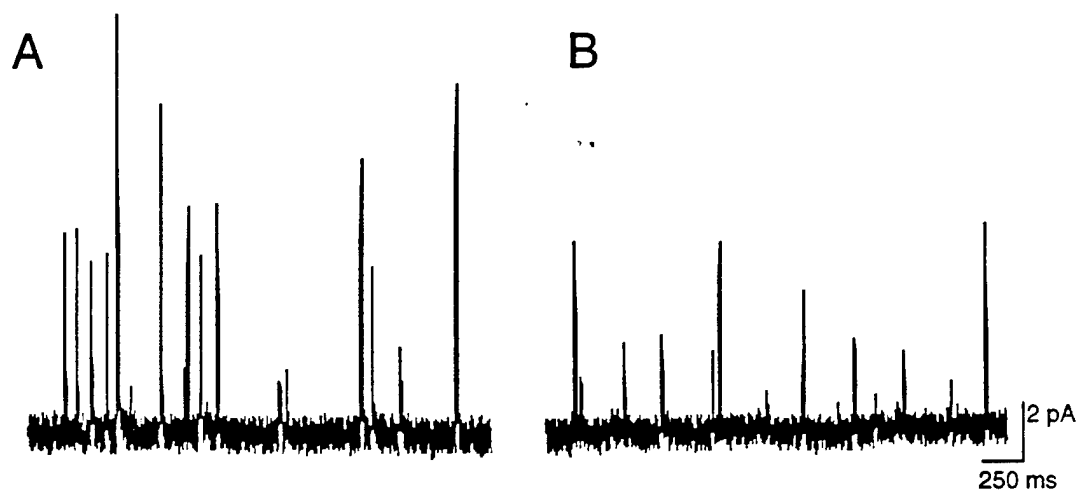
Mean ± s.e.m. (n=3) for DA (fmol) peaks in single GDCs from juvenile *Planorbis corneus* measured by capillary electrophoresis. Cells were exposed to 10 μ M AMPH for 15 min prior to sampling. The detection limit is typically 1 to 10 amol.



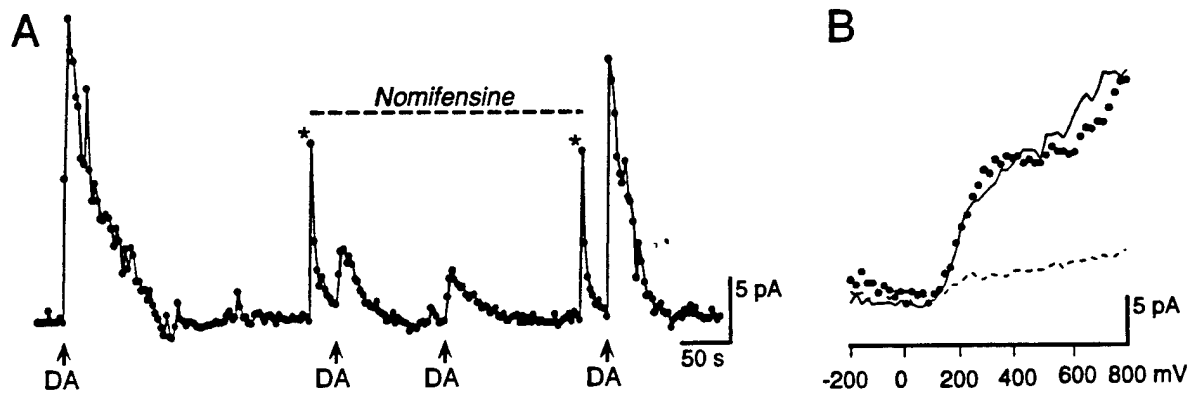
Sulzer Fig 1
One column width



Sulzer Fig 2
One column width



Sulzer Fig 3
Two column width



Sulzer Fig 4
Two column width